TITLE

DIPEPTIDYL PEPTIDASES

FIELD OF INVENTION

The invention relates to a dipeptidyl peptidase, to a nucleic acid molecule which encodes it, and to uses of the peptidase.

BACKGROUND OF THE INVENTION

The dipeptidyl peptidase (DPP) IV-like gene family is a family of molecules which have related protein structure and function [1-3]. The gene family includes the following molecules: DPPIV (CD26), dipeptidyl amino-peptidase-like protein (DPP6) and fibroblast activation protein (FAP) [1,2,4,5]. Another possible member is DPPIV-β[6].

The molecules of the DPPIV-like gene family are serine proteases, they are members of the peptidase family S9b, and together with prolyl endopeptidase (S9a) and acylaminoacyl peptidase (S9c), they are comprised in the prolyl oligopeptidase family[5,7].

DPPIV and FAP both have similar postproline dipeptidyl amino peptidase activity, however, unlike DPPIV, FAP also has gelatinase activity[8,9].

DPPIV substrates include chemokines such as RANTES, eotaxin, macrophage-derived chemokine and stromal-cell-derived factor 1; growth factors such as glucagon and glucagon-like peptides 1 and 2; neuropeptides including neuropeptide Y and substance P; and vasoactive peptides[10-12].

DPPIV and FAP also have non-catalytic activity; DPPIV binds adenosine deaminase, and FAP binds to $\alpha_3\beta_1$ and $\alpha_5\beta_1$ integrin[13-14].

In view of the above activities, the DPPIV-like family members are likely to have roles in intestinal and renal handling of proline containing peptides, cell adhesion, peptide metabolism, including metabolism of cytokines, neuropeptides, growth factors and chemokines, and immunological processes, specifically T cell stimulation[3,11,12].

Consequently, the DPPIV-like family members are likely to be involved in the pathology of disease, including for example, tumour growth and biology, type II diabetes, cirrhosis, autoimmunity, graft rejection and HIV infection[3,15-18].

Inhibitors of DPPIV have been shown to suppress arthritis, and to prolong cardiac allograft survival in animal models *in vivo*[19,20]. Some DPPIV inhibitors are reported to inhibit HIV infection[21]. It is anticipated that DPPIV inhibitors will be useful in other therapeutic applications including treating diarrhoea, growth hormone deficiency, lowering glucose levels in non insulin dependent diabetes mellitus and other disorders involving glucose intolerance, enhancing mucosal regeneration and as immunosuppressants[3,21-24].

There is a need to identify members of the DPPIV-like gene family as this will allow the identification of inhibitor(s) with specificity for particular family member(s), which can then be administered for the purpose of treatment of disease. Alternatively, the identified member may of itself be useful for the treatment of disease.

SUMMARY OF THE INVENTION

The present invention seeks to address the above identified need and in a first aspect provides a peptide which comprises the amino acid sequence shown in SEQ ID NO:1.

This peptide has substrate specificity for the following compounds: H-Ala-Pro-pNA, H-Gly-Pro-pNA and H-Arg-Pro-pNA. Therefore, it is a prolyl oligopeptidase and a dipeptidyl peptidase, because it is capable of hydrolysing the peptide bond C-terminal to proline in each of these compounds.

The peptide is homologous with human DPPIV, and importantly, identity between the sequences of DPPIV and SEQ ID NO: 1 is observed at the region of DPPIV containing the catalytic triad residues and the two glutamate residues of the β-propeller domain essential for DPPIV enzyme activity. The observation of amino acid sequence homology means that the peptide which has the amino acid sequence shown in SEQ ID NO:1 is a member of the DPPIV-like gene family. Accordingly the peptide was provisionally named DPPIVL1, and is now named and described herein as DPP8.

The following sequences of the human DPPIV amino acid sequence are important for the catalytic activity of DPPIV: (i) Tyr^{627}GlyTrpSerTyrGlyGlyTyrVal; (ii) Ala^{707}AspAspAsnValHisPhe; (iii) Glu^{738}AspHisGlyIleAlaGln; and (iv) Tyr^{201}ValTyrGluGluGluVal [25-28]. As described herein, the alignment of the following sequences of DPP8: His^{736}GlyTrpSerTyrGlyGlyTyrLeu; Leu^{816}AspGluAsnValHisPheAla; Glu^{847}ArgHisSerIleArg and Phe^{255}ValLeuGlnGluGluPhe with sequences (i) to (iv) above, respectively, suggests that these sequences of DPP8 are likely to confer the catalytic activity of DPP8. Thus, in a second aspect, the invention provides a peptide comprising the following amino acid

sequences: His^{736}GlyTrpSerTyrGlyGlyTyrLeu; Leu^{816}AspGluAsnValHisPheAlaHis; Glu^{847}ArgHisSerIleArg and Phe^{255}ValLeuGlnGluGluPhe; which has the substrate specificity of the sequence shown in SEQ ID NO:1.

Also described herein, using multiple sequence alignment, it is observed that DPP8 has 55% amino acid similarity and 32% amino acid identity with a *C. elegans* protein. Further, as shown herein, a nucleic acid molecule which encodes DPP8, is capable of hybridising specifically with DPP8 sequences derived from non-human species. Together these data suggest that DPP8 is expressed in non-human species. Thus in a third aspect, the invention provides a peptide which has at least 60% amino acid identity with the amino acid sequence shown in SEQ ID NO:1, and which has the substrate specificity of the sequence shown in SEQ ID NO:1. Preferably, the amino acid identity is 75%. More preferably, the amino acid identity is 95%. Amino acid identity is calculated using GAP software [GCG Version 8, Genetics Computer Group, Madison, WI, USA] as described further herein. Typically, the non-human DPP8 comprises the following sequences: His^{736}GlyTrpSerTyrGlyGlyTyrLeu; Leu^{816}AspGluAsnValHisPheAlaHis; Glu^{847}ArgHisSerIleArg and Phe^{255}ValLeuGlnGluGluPhe.

In view of the homology between DPPIV and DPP8 amino acid sequences, it is expected that these sequences will have similar tertiary structure. This means that the tertiary structure of DPP8 is likely to include the seven-blade β-propeller domain and the α/β hydrolase domain of DPPIV. These structures in DPP8 are likely to be conferred by the regions comprising β-propeller, Gly^{180} to Asp^{606}, α/β hydrolase, Ser^{607} to Ile^{882} and about 70 to 100 residues in the region Arg^{39} to Gln^{179}. As it is known that the β-propeller domain regulates proteolysis mediated by the catalytic triad in the α/β hydrolase domain of prolyl

oligopeptidase, [29] it is expected that truncated forms of DPP8 can be produced, which have the substrate specificity of the sequence shown in SEQ ID NO:1, comprising the regions referred to above (His^{736}GlyTrpSerTyrGlyGlyTyrLeu; Leu^{816}AspGluAsnValHisPheAlaHis; Glu^{847}ArgHisSerIleArg and Phe^{255}ValLeuGlnGluGluPhe) which confer the catalytic specificity of DPP8. Examples of truncated forms of DPP8 which might be prepared are those in which the region conferring the β-propeller domain and the α/β hydrolase domain are spliced together. Other examples of truncated forms include those which are encoded by splice variants of DPP8 mRNA. Thus although, as described herein, the biochemical characterisation of DPP8 shows that DPP8 consists of 882 amino acids and has a molecular weight of about 100kDa, it is recognised that truncated forms of DPP8 which have the substrate specificity of the sequence shown in SEQ ID NO:1, may be prepared using standard techniques [30,31]. Thus in a fourth aspect, the invention provides a fragment of the sequence shown in SEQ ID NO: 1, which has the substrate specificity of the sequence shown in SEQ ID NO:1. Preferably, the fragment has an amino acid sequence shown in SEQ ID NO: 3, 5 or 7.

As described herein, the sequence shown in SEQ ID NO:1 does not contain a consensus sequence for N-linked glycosylation. Therefore it is unlikely that DPP8 is associated with N-linked glycosylation. In this regard, DPP8 is distinguished from other DPPIV-like gene family members, which contain between 6 and 9 consensus sequences for N-linked glycosylation. Thus in one embodiment, an asparagine residue in the peptide of the first aspect of the invention is not linked to a carbohydrate molecule.

The analysis of DPP8 expression described herein shows that it is likely that DPP8 is expressed as a cytoplasmic protein. The expression of DPP8 is therefore

distinguished from other DPPIV-like gene family members, which are expressed on the cytoplasmic membrane, or in other words, the cell surface membrane. Thus in another embodiment, the peptide of the first aspect of the invention is not expressed on a cell surface membrane of a cell.

It is recognised that DPP8 may be fused, or in other words, linked to a further amino acid sequence, to form a fusion protein which has the substrate specificity of the sequence shown in SEQ ID NO:1. An example of a fusion protein is described herein which comprises the sequence shown in SEQ ID NO:1 which is linked to a further amino acid sequence: a "tag" sequence which consists of an amino acid sequence encoding the V5 epitope and a His tag. An example of another further amino acid sequence which may be linked with DPP8 is a glutathione S transferase (GST) domain [30]. Another example of a further amino acid sequence is a portion of CD8α [8]. Thus in one aspect, the invention provides a fusion protein comprising the amino acid sequence shown in SEQ ID NO:1 linked with a further amino acid sequence, the fusion protein having the substrate specificity of the sequence shown in SEQ ID NO:1.

It is also recognised that the peptide of the first aspect of the invention may be comprised in a polypeptide, so that the polypeptide has the substrate specificity of DPP8. The polypeptide may be useful, for example, for altering the protease susceptibility of DPP8, when used in *in vivo* applications. An example of a polypeptide which may be useful in this regard, is albumin. Thus in another embodiment, the peptide of the first aspect is comprised in a polypeptide which has the substrate specificity of DPP8.

As described above, the isolation and characterisation of DPP8 is necessary for identifying inhibitors of DPP8 catalytic activity, which may be useful for the treatment of disease. A method for identifying inhibitors of DPP8 catalytic activity, described herein, has identified that various inhibitors of DPPIV and serine proteases, zinc and mimetic peptides, Ala-Pro-Gly and Lys-Pro, but not inhibitors of metalloproteinases, aspartyl proteinases or cysteinyl proteinases, inhibit DPP8 catalytic activity. Accordingly, in a fifth aspect, the invention provides a method of identifying a molecule capable of inhibiting cleavage of a substrate by DPP8, the method comprising the following steps:

(a) contacting DPP8 with the molecule;

(b) contacting DPP8 of step (a) with a substrate capable of being cleaved by DPP8, in conditions sufficient for cleavage of the substrate by DPP8; and

(c) detecting substrate not cleaved by DPP8, to identify that the molecule is capable of inhibiting cleavage of the substrate by DPP8.

It is recognised that although inhibitors of DPP8 may also inhibit DPPIV and other serine proteases, as described herein, the alignment of the DPP8 amino acid sequence with most closely related molecules,(i.e. DPPIV), reveals that the DPP8 amino acid is distinctive, particularly at the regions controlling substrate specificity. Accordingly, it is expected that it will be possible to identify inhibitors which inhibit DPP8 catalytic activity specifically, which do not inhibit catalytic activity of DPPIV-like gene family members, or other serine proteases. Thus, in a sixth aspect, the invention provides a method of identifying a molecule capable of inhibiting specifically, the cleavage of a substrate by DPP8, the method comprising the following steps:

(a) contacting DPP8 and a further protease with the molecule;

(b) contacting DPP8 and the further protease of step (a) with a substrate capable of being cleaved by DPP8 and the further protease, in conditions sufficient for cleavage of the substrate by DPP8 and the further protease; and

(c) detecting substrate not cleaved by DPP8, but cleaved by the further protease, to identify that the molecule is capable of inhibiting specifically, the cleavage of the substrate by DPP8.

In a seventh aspect, the invention provides a method of reducing or inhibiting the catalytic activity of DPP8, the method comprising the step of contacting DPP8 with an inhibitor of DPP8 catalytic activity. As various inhibitors of DPPIV catalytic activity are shown herein to inhibit DPP8 catalytic activity, it is recognised that other inhibitors of DPPIV may be useful for inhibiting DPP8 catalytic activity. Examples of inhibitors suitable for use in the seventh aspect are described in [21,32,33]. Other inhibitors useful for inhibiting DPP8 catalytic activity can be identified by the methods of the fifth or sixth aspects of the invention, which methods are exemplified herein.

In one embodiment, the catalytic activity of DPP8 is reduced or inhibited in a mammal by administering the inhibitor of DPP8 catalytic activity to the mammal. It is recognised that these inhibitors have been used to reduce or inhibit DPPIV catalytic activity *in vivo*, and therefore, may also be used for inhibiting DPP8 catalytic activity *in vivo*. Examples of inhibitors useful for this purpose are disclosed in the following [21,32-34].

Preferably, the catalytic activity of DPP8 in a mammal is reduced or inhibited in the mammal, for the purpose of

treating a disease in the mammal. Diseases which are likely to be treated by an inhibitor of DPP8 catalytic activity are those in which DPPIV-like gene family members are associated [3,10,11,17,21,36], including for example, neoplasia, type II diabetes, cirrhosis, autoimmunity, graft rejection and HIV infection.

Preferably, the inhibitor for use in the seventh aspect of the invention is one which inhibits the cleavage of a peptide bond C-terminal adjacent to proline. As described herein, examples of these inhibitors are 4-(2-aminoethyl)benzenesulfonylfluoride, aprotinin, benzamidine/HCl, Ala-Pro-Gly, H-Lys-Pro-OH HCl salt and zinc ions, for example, zinc sulfate or zinc chloride. More preferably, the inhibitor is one which specifically inhibits DPP8 catalytic activity, and which does not inhibit the catalytic activity of other serine proteases, including, for example DPPIV or FAP.

In an eighth aspect, the invention provides a method of cleaving a substrate which comprises contacting the substrate with DPP8 in conditions sufficient for cleavage of the substrate by DPP8, to cleave the substrate. Examples of molecules which can be cleaved by the method are H-Ala-Pro-pNA, H-Gly-Pro-pNA and H-Arg-Pro-pNA. The conditions sufficient for cleaving the substrate are described herein. Molecules which are cleaved by DPPIV including RANTES, eotaxin, macrophage-derived chemokine, stromal-cell-derived factor 1, glucagon and glucagon-like peptides 1 and 2, neuropeptide Y, substance P and vasoactive peptide are also likely to be cleaved by DPP8 [11,12]. In one embodiment, the substrate is cleaved by cleaving a peptide bond C-terminal adjacent to proline in the substrate. The molecules cleaved by DPP8 may have Ala, or Trp, Ser, Gly, Val or Leu in the P1 position, in place of Pro [11,12].

As described herein, DPP8 gene expression is upregulated in stimulated lymphocyte and lymphocytic cell lines which suggests that DPP8 may have a functional role in T cell costimulation and proliferation. It is recognised therefore that measuring DPP8 gene expression is useful for detecting T cell activation. Thus in a ninth aspect, the invention provides a method of detecting an activated T cell, the method comprising the step of detecting the level of DPP8 gene expression in a T cell. In one embodiment, the level of DPP8 gene expression is detected by measuring the amount of DPP8 mRNA in the cell, as described herein.

The inventors have characterised the sequence of a nucleic acid molecule which encodes the amino acid sequence shown in SEQ ID NO:1. Thus in a tenth aspect, the invention provides a nucleic acid molecule which encodes the amino acid sequence shown in SEQ ID NO:1.

In an eleventh aspect, the invention provides a nucleic acid molecule which consists of the sequence shown in SEQ ID NO:2.

As described herein, at least three splice variants of DPP8 RNA which have an open reading frame from 2.6 to 3.1 kb in length are observed. As a frame shift mutation or termination signal was not observed in the sequence of these splice variants, and as the coding sequence of two of the splice variants includes a sequence which encodes the amino acid sequence associated with catalytic activity, it is recognised that some of the peptides encoded by the splice variants are likely to have the substrate specificity of DPP8. Thus in an embodiment, the nucleic acid molecule is a fragment of the sequence shown in SEQ ID NO: 1 which is about 2.6 to 3.1 kb in length and which encodes a peptide which has the substrate

specificity of the sequence shown in SEQ ID NO:1. Preferably, the nucleic acid molecule has a sequence shown in any one of SEQ ID NO.s: 4, 6 and 8.

In a twelfth aspect, the invention provides a nucleic acid molecule which is capable of hybridising to a nucleic acid molecule consisting of the sequence shown in SEQ ID NO:2 in stringent conditions, and which encodes a peptide which has the substrate specificity of the sequence shown in SEQ ID NO:1. As shown in the Northern blot analysis described herein, DPP8 mRNA hybridises specifically to the sequence shown in SEQ ID NO:2, after washing in 2XSSC/ 1.0%SDS at 37°C, or after washing in 0.1XSSC/0.1% SDS at 50°C. "Stringent conditions" are conditions in which the nucleic acid molecule is exposed to 2XSSC/ 1.0% SDS. Preferably, the nucleic acid molecule is capable of hybridising to a molecule consisting of the sequence shown in SEQ ID NO:2 in high stringent conditions. "High stringent conditions" are conditions in which the nucleic acid molecule is exposed to 0.1XSSC/ 0.1%SDS at 50°C.

As described herein, the inventors believe that the gene which encodes DPP8 is located at band q22 on human chromosome 15. The location of the DPP8 gene is distinguished from genes encoding other prolyl oligopeptidases, which are located on chromosome 2, at bands 2q24.3 and 2q23, or chromosome 7. Thus in an embodiment, the nucleic acid molecule is one capable of hybridising to a gene which is located at band q22 on human chromosome 15.

It is recognised that a nucleic acid molecule which encodes the amino acid sequence shown in SEQ ID NO:1, or which comprises has the sequence shown in SEQ ID NO:2, could be made by producing the fragment of the sequence which is translated, using standard techniques [30,31].

Thus in an embodiment, the nucleic acid molecule does not contain 5′ or 3′ untranslated sequences.

In a thirteenth aspect, the invention provides a vector which comprises a nucleic acid molecule of the tenth aspect of the invention. In one embodiment, the vector is capable of replication in a COS-7 cell, CHO cell or 293T cell, or E.coli. In another embodiment, the vector is selected from the group consisting of λTripleEx, pTripleEx, pGEM-T Easy Vector, pSecTag2Hygro, pet15b, pEE14.HCMV.gs and pCDNA3.1/V5/His.

In a fourteenth aspect, the invention provides a cell which comprises a vector of the thirteenth aspect of the invention. In one embodiment, the cell is an E.coli cell. Preferably, the E. coli is MC1061, DH5α, JM109, BL21DE3, pLysS. In another embodiment, the cell is a COS-7, COS-1, 293T or CHO cell.

In a fifteenth aspect, the invention provides a method for making a peptide of the first aspect of the invention comprising, maintaining a cell according to the fourteenth aspect of the invention in conditions sufficient for expression of the peptide by the cell. The conditions sufficient for expression are described herein. In one embodiment, the method comprises the further step of isolating the peptide.

In a sixteenth aspect, the invention provides a peptide when produced by the method of the fifteenth aspect.

In a seventeenth aspect, the invention provides a composition comprising a peptide of the first aspect and a pharmaceutically acceptable carrier.

In an eighteenth aspect, the invention provides an antibody which is capable of binding a peptide according to the first aspect of the invention. The antibody can be prepared by immunising a subject with purified DPP8 or a fragment thereof according to standard techniques [35]. As described herein, an antibody was prepared by immunising with transiently transfected DPP8$^+$ cells. It is recognised that the antibody is useful for inhibiting activity of DPP8, or for detecting increased gene expression of DPP8, for the purpose of identifying an activated T cell. In one embodiment, the antibody of the eighth aspect of the invention is produced by a hybridoma cell.

In a nineteenth aspect, the invention provides a hybridoma cell which secretes an antibody of the nineteenth aspect.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Cloning strategy for isolating full-length DPP8 cDNA and the alternative splicing variants of DPP8 observed. Representation of three splice variants is shown including loss of serine recognition site by one splice variant (T8).

Figure 2. Nucleotide sequence and amino acid sequence of human DPP8. The nucleotide and predicted one letter code amino acid sequence are shown. This sequence shows no putative membrane spanning domain (deduced from hydrophobicity plots) or potential N-linked glycosylation sites. The putative serine recognition site and aspartic acid and histidine which form the Ser-Asp-His catalytic triad are marked. Base pairs are numbered in the right margin.

Figure 3. Alignment of the deduced amino acid residue sequence of DPP8 with the *C. elegans* homolog of DPP8 and human DPPIV. Amino-acid residues are numbered in the right margin. Amino-acid residues identical in all three proteins are boxed. Asterisks mark the putative catalytic triad residues and two glutamates of the β-propeller domain essential for DPPIV enzyme activity. The grey shading denotes the α/β hydrolase domain of these proteins. Filled triangles joined by lines indicate starts and ends of alternatively spliced transcripts, stPBMCdy3-3-10 (solid lines), T8 (dashed lines) and T21 (solid lines). The alignment was constructed using the PILEUP program in GCG.

Figure 4. Northern Blot analysis of DPP8 expression. Human multiple tissue Northern blots (CLONTECH) containing 2 μg per lane of poly A^+ RNA were hybridized with a ^{32}P labeled DPP8 probe at 68°C and washed at high stringency. The autoradiograph was exposed for 1 day at −70°C with a BIOMAX MS screen. Molecular mass markers are indicated in base pairs on the left side of each autoradiogram. Figure 4a. Master RNA (CLONTECH) blot of poly A^+ RNA was hybridized with a ^{32}P labelled DPP8 probe at 65°C and washed at high stringency. The autoradiograph was exposed for 3 days at −70°C with BIOMAX MS screen. DPP8 mRNA was detected in all tissues examined.

Figure 5. Chromosomal localization of human DPP8. Metaphase showing FISH with the biotinylated DPP8 cDNA probe. Normal male chromosomes stained with DAPI. Hybridization sites on chromosome 15 are indicated by an arrow.

Figure 6. Western blot analysis of transfected cell lines. Analysis of lysates of stable cell lines. DPP8 protein was

seen in DPP8 /V5/His stable cell lines but not in DPP4 or vector-only stable cell lines. The electrophoretic mobility of the protein was not altered when samples were boiled. The band of greater mobility was probably a breakdown product of intact DPP8.

Figure 7. DPP8 enzyme activity. (A) pH-dependence of DPP8 enzyme activity. (B) DPP8 and DPPIV enzyme kinetics. Means +/- SD of absorbance change per minute, multiplied by 1000 are shown. Curve fitting assumed Michaelis-Menten kinetics.

Figure 8. RT-PCR analysis of DPP8 expression. PCR amplifications with primers specific for either a portion of human DPP8 that contained no alternate splicing, Val416 to Gly 679 (top of each gel) or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (bottom of each gel. (A) Top gel, lanes 1-5 contain PCR products from unstimulated PBMC cDNA from five subjects. Bottom gel, lanes 6 to 11 contain PCR products from OKT3-stimulated PBMC cDNA from six subjects. (B). PCR products are from cDNA from lymphocytic cell lines, liver or placenta as indicated. Negative control amplifications contained reaction mix, enzyme and no cDNA template. Each PCR was performed for 35 cycles. The PCR products were electrophoresed on agarose gels and stained with ethidium bromide. The left lane of each gel contains PUC19 digested with *Hae*III as size markers.

Figure 9. Northern blot analysis of murine DPP8 expression. A murine Northern blot containing 10 μg per lane of total RNA was hybridized with a ^{32}P-labeled human DPP8 probe at 60°C and washed at low stringency. Autoradiographic exposure was for 3 days at -70°C with a BIOMAX MS screen.

DETAILED DESCRIPTION OF THE INVENTION

EXAMPLES

General

Restriction enzymes and other enzymes used in cloning were obtained from Boehringer Mannheim Roche. Standard molecular biology techniques were used [31] unless indicated otherwise.

An EST clone (GENBANK™ accession number AA417787) was obtained from American Type Culture Collection. The DNA insert of this clone was sequenced on both strands using automated sequencing at SUPAMAC (Sydney, Australia).

Cell culture and RNA preparation

Human peripheral blood monocytes (PBMCs) were isolated by Ficoll-Hypaque density-gradient centrifugation (Pharmacia, Uppsala, Sweden) of blood obtained from healthy donors. The PBMCs were incubated in AIM-V medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 2 mM L-glutamine and were stimulated with either 1 $\mu g.mL^{-1}$ phytohaemagglutinin (Wellcome) or 100ng.mL^{-1} OKT3 (Orthoclone, FL, USA) for 72 h. The human cell lines Jurkat, CCRF-CEM, Raji, Daudi and HepG2 were grown to confluence in Dulbecco's modified Eagle's medium (Trace Biosciences, NSW, Australia) supplemented with 10% fetal bovine serum and 2mM L-glutamine.

Liver and placental RNA were prepared from snap-frozen human tissue as described previously [37]. However, RNA was prepared from PBMCs and cell lines using an RNAeasy kit (Qiagen, Germany).

Bioinformatics

BLAST programs [38] and all multiple sequence alignments

were performed through the Australian National Genomic Information Service (ANGIS, Sydney, NSW, Australia). PILEUP (GCG Version 8, Genetics Computer Group, Madison, WI, USA) was used for multiple sequence alignments of proteins.

A BLAST search was performed on the public expressed sequence tag (EST) database using the complete human DPPIV (GenBank™ accession number X60708) and FAP (accession number U09278) nucleotide sequences as query sequences. An EST clone (accession number AA417787) was obtained from the American Type Culture Collection. The DNA insert of this clone was sequenced on both strands using automated sequencing at SUPAMAC (Sydney, NSW, Australia). Because of its homology with DPPIV, this new gene was named dipeptidyl peptidase 8 (DPP8).

DPP8 Cloning

ESTAA417787 was used to design forward (caa ata gaa att gac gat cag gtg) and reverse (tct tga agg tag tgc aaa aga tgc) DPP8 primers for polymerase chain reaction (PCR) from ESTAA417787. The PCR conditions were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 1 minute, 55°C for 30 sec and 70°C for 1 min. This 484 bp PCR product was gel purified, ^{32}P-α labelled using Megaprime Labeling Kit (Amersham Pharmacia Biotec, UK) and hybridized to a Master RNA blot (CLONTECH, Palo Alto, CA, USA) that contained poly A^{+} from 50 adult and fetal tissues immobilized in dots as per manufacturers' instructions. This Master RNA blot was also probed with DPP4 for comparison of mRNA tissue expression.

The forward and reverse DPP8 primers were used for PCR to screen a human placental λ STRETCH PLUS library (CLONTECH, Palo Alto, CA, USA) for the presence of DPP8 cDNA in the

library. The library was then screened by standard molecular biology techniques [30,31]. After primary screening, 23 clones were selected for secondary screening, after which 22 remained positive. For the tertiary screen the clones contained in λTripleEx were converted into pTriplEx plasmids and transformed into BM25.8 *E. coli* recipient bacteria. The plated bacteria were screened and it was confirmed that all 22 clones were positive. Two of these clones, T8 and T21 were selected for further study.

5'RACE (Rapid amplification of cDNA ends)

A 5' RACE Version 2.0 kit (Gibco BRL, Life technologies) was applied on activated T cell (ATC) and placental RNA as prescribed in the kit instructions. The T8 DNA sequence was used to design GSP 1 (TCC TTC CTT CAG CAT CAA TC) and GSP2 (CTT AAA AGT GAC TTT AGG ATT TGC TGT ACC). 5' RACE PCR products were cloned into pGEM-T Easy®Vector (Promega Co., Madison, WI, USA) and sequenced by primer walking.

Confirmation of identity of RACE product

Reverse transcriptase PCR was carried out on ATC RNA using DPP8-pr23 (GGA AGA AGA TGC CAG ATC AGC TGG) and DPP8-pr19r (TCC GTG TAT CCT GTA TCA TAG AAG) to span across the junction between the RACE product and the EST and library clones. Two gel purified products ATCd3-2-1 (1603bp) and ATC3-3-10 (1077bp) were cloned into pGEM-T Easy® (Promega Co., Madison, WI, USA) and sequenced.

Subcloning of DPP8 cDNA into a pcDNA3.1/V5/His Expression Vector

The ATC RACE product, the ATCd3-2-1 (1603bp) junction fragment and the library clone T21 were joined together and cloned into the expression vector pcDNA3.1/V5/His A (Invitrogen, the Netherlands) to form a DPP8 cDNA of 3.1

kb with an open reading frame of 882 aa. The first construct was made using three sequential cloning steps. Firstly, a *Eco RV/Xba I* fragment of T21 (containing 3' DPP8, stop codon and 3' untranslated region on DPP8 cDNA) was ligated into the vector pcDNA3.1/V5/His A which had been digested with *Eco RV/Xba I*. An *Eco RI/Eco RV* fragment of ATCd3-2-1 was then added to this construct digested with *Eco RI/Eco RV*. Finally the RACE product was cut with *Eco RI* and cloned into the *Eco RI* site of the previous construct to form the complete 3.1 kb DPP8 cDNA. This construct pcDNA3.1-DPP8 expressed protein with no detectable tag. In addition the stop codon in the DPP8 expression construct in pcDNA3.1/V5/His V5 was genetically altered using PCR to create a C-terminal fusion with the V5 and His tag contained in the vector. This construct was named pcDNA3.1- DPP8/V5/His. All expression constructs subcloned into pcDNA3.1/V5/His were verified by full sequence analysis.

DPP8 gene expression by Northern Blot

Human multiple tissue Northern blots (CLONTECH) containing 2 ug of poly A$^+$ RNA were prehybridized in Express Hybridization solution (CLONTECH) for 30 min at 68°C. Both the DPP8 484 bp product and the 5' RACE ATC product were radiolabeled using a Megaprime Labeling kit (Amersham Pharmacia Biotech) and [^{32}P]dCTP (NEN Dupont). Unincorporated label was removed using a NICK column (Amersham Pharmacia Biotech) and the denatured probe was incubated for 2 hrs at 68°C in Express Hybridization solution. Washes were performed at high stringency and blots exposed to BIOMAX MS film for overnight with a BIOMAX MS screen at -70°C.

DPP8 gene expression in mice by Northern Blot

A Northern blot containing 10 ug of total liver RNA per lane was made using standard methods [31]. The RNA was derived from male and female mice of two strains, C57Bl6 and Balb/c. The Northern blot was prehybridized in Express Hybridization solution (CLONTECH, Palo Alto, USA) for 1 hr at 60°C. A 2.4kb human DPP8 cDNA (PCR product) was radiolabeled using the Megaprime Labeling kit (Amersham Pharmacia Biotech) and [^{32}P]dCTP (NEN Dupont). Unincorporated label was removed using a NICK column (Amersham Pharmacia Biotech) and the denatured probe was incubated with the blot overnight at 60°C in Express Hybridization solution. Washes were performed at low stringency (2 x SSC/0.05% SDS for 1 h at 37°C followed by 0.1x SSC/0.1% SDS for 30 min at 40°C) and blots exposed to BIOMAX MS film for three days with a BIOMAX MS screen at -70°C.

Expression of DPP8 in mouse liver using rtPCR

Mouse liver RNA was reverse transcribed using the Superscript II enzyme kit (Gibco BRL, Gaithersburg, MD) as described previously [42]. The cDNA was diluted 1 in 4 and stored in aliquots at - 70°C. PCR using mouseDPP8-pr1F (atg att acc acc cag gaa gcg) as the forward primer and mouseDPP8-pr2R (atc tcc gac atc ttg aaa gtg acc) as the reverse primer was used to detect mouse DPP8 mRNA.

One ul of diluted cDNA was amplified in a 50 ul PCR reaction which contained: 0.2 mM dNTPs, 1 ul of 50 x Advantage 2 Polymerase Mix (Clontech), 1 X Advantage 2 PCR buffer (Clontech) and 100 ng of each primer. The PCR involved an initial step of 95°C for 1 min to inactivate the TaqStart Antibody. This was followed by 35 cycles; denaturation at 95°C for 30 sec, 68°C for 1 min, followed by a final step of 68°C for 1 min. The amplified products were analysed by electrophoresis of 10 μl of PCR reaction on a 3:1 Nusieve gel (FMC Bioproducts, Rockville, MD) plus

0.5 μg/ml ethidium bromide in TAE buffer (0.04M Tris acetate, 0.001 M EDTA, pH 8.0). The gel was then Southern Blotted using standard techniques [31]. The Southern blot was hybridized at 60°C for 2hr with the 2.4 kb human DPP8 cDNA probe prepared as described above. Washes were performed at low stringency (2 x SSC/0.05% SDS for 1 h at 37°C followed by 0.1x SSC/0.1% SDS for 40 min at 50°C). The blot was exposed to XAR5 Kodak film for 30 min at RT.

DPP8 expression by RT-PCR

Reverse transcriptase PCR was performed on human ATC RNA, human placental RNA and human liver RNA using TED primers DPP8/pr3 (GCA CTA CCT TCA AGA AAA CCT TGG) and DPP8/pr20R (TAT GGT ATT GCT GGG TCT CTC AGG) to give a 293 bp product.

Transfection, Western blot, immunocytochemistry, cytochemistry and flow cytometry

Monkey kidney fibroblast (COS-7) cells (American Type Culture Collection, CRL-1651) were grown and transfected as described previously [39]. For making stable cell lines, Geneticin (G418; Gibco-BRL) was added to the medium, beginning 24 h after transfection.. COS cell extracts were prepared by sonication followed by differential centrifugation and neither boiled nor reduced before SDS/PAGE (10% gel) and transfer to nitrocellulose, as described previously [40,9]. The presence of DPP8 fused with the V5 epitope was detected using an anti-V5 mAb (Invitrogen). COS cell monolayers were fixed in cold ethanol before staining with anti-V5 mAb [39,41,9]. Some monolayers were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 [35], then double-stained with wheat germ agglutinin to label Golgi apparatus and with goat anti-mouse IgG to label DPP8, conjugated to Alexa Fluor 488 and Alexa Fluor 594,

respectively (Molecular Probes, Eugene, OR, USA). Flow cytometry and confocal scanning microscopy using a Leica TCS-NT confocal microscope have been described previously [39,9].

Purification of recombinant DPP8/V5/His and DPPIV/V5/His

Cells (1 x 10^7) expressing each protein were sonicated in native buffer (50mM sodium phosphate, 300 mM NaCl), then treated with 700 U DNAse for 20 min at room temperature. DPPIV is expressed at the cell surface, so 1% Triton X-100 was used to solubilize DPPIV/V5/His. Insoluble material was removed by centrifugation. The supernatant was incubated with 1 mL Talon® Metal Affinity Resin (Clontech) following the manufacturer's instructions for a batch/gravity flow procedure. The resin was washed with 50 mM sodium phosphate, containing 300 mM NaCl and 5 mM imidazole, and proteins were eluted using the same buffer containing 150 mM imidazole. Enzyme activity was used to monitor eluted fractions.

Enzyme assays

Enzyme assays were performed as described previously [1]. Either clarified cell extract from 1 x 10^4 sonicated COS-7 cells or purified protein derived from 1 x 10^5 cells was incubated with substrate in 70μL phosphate buffer, pH 7.4, for 30 min at 37°C, except where otherwise indicated. The specific DPPIV substrates, Gly-Pro-toluenesulfonate, H-Gly-Pro-*p*-nitroanilide (NA)/HCl (Sigma, St Louis, MO, USA) and Gly-Pro-7-amino-4-trifluromethylcoumarin (Calbiochem, San Diego, CA, USA) were tested. Other substrates tested were H-Ala-Pro-pNA/HCl, H-Arg-Pro-pNA acetate salt, H-Lys-Ala-pNA.2HCl, H-Asp-Pro-pNA, H-Ala-Ala-pNA/HCl, H-Ala-Ala-Pro-pNA/HCl, H-Ala-Ala-Phe-pNA, succinyl-Ala-Pro-pNA, H-Ala-Phe-Pro-pNA and Z-Ala-Pro-p-NA from Bachem

(Switzerland). H-Ala-Pro-4-methoxyβNA/HCl, Z-Lys-Pro-4-methoxyβNAformate salt, H-Lys-Pro-4-methoxyβNA/HCl, Z-Ala-Pro-4-methoxyβNA, H-Gly-Pro-βNA and H-His-Ser-4-methoxyβNAacetate salt (Bachem) were tested for their ability to stain unfixed transfected cells.

All inhibitors were (see Table 2) incubated with each purified enzyme in phosphate buffer, pH 7.4, for 15 min before the addition of substrate. After the addition of 1mM H-Ala-Pro-pNA substrate for purified DPP8 and 1 mM H-Gly-Pro-pNA substrate for purified DPPIV, samples were incubated for 60 min at 37°C. All enzyme assays were performed in triplicate.

<u>Chromosomal localization of DPP8 by Fluorescence in situ Hybridization (FISH) analysis</u>

DPP8 was localized using two different probes, the DPP8 EST and the T8 clone. The probes were nick-translated with biotin-C^{14}-dATP and hybridized *in situ* at a final concentration of 10ng/ul to metaphases from two normal males. The FISH method was modified from that previously described [37] in that chromosomes were stained before analysis with both propidium iodide (as counterstain) and DAPI (for chromosomal identification). Images of metaphase preparations were captured by a cooled CCD camera using the Cyto Vision Ultra image collection and enhancement system (Applied Imaging International Ltd). FISH signals and the DAPI banding pattern were merged for figure preparation.

<u>Expression of DPP8 in human lymphocytes and cell lines</u>

RNA (1μg) was reverse-transcribed using the Superscript II enzyme kit (Gibco-BRL) as described previously [42]. PCR using DPP8-pr18 (CTGTGACGCCACTAATTATCTATG) as the forward primer and DPP8-pr26R (CCTAGAGAGGCTAGGGTATTCAAG) as the

reverse primer was used to detect full-length DPP8 mRNA. The glyceraldehyde-3-phosphate dehydrogenase (G3PDH) control primer set was G3PDH for (ACCACAGTCCATGCCATCAC) and G3PDHrev (TCCACCACCCTGTTGCTGTA) to give a 470-bp product.

cDNA (diluted 1 : 4; 1μg) was amplified in a 25-μL PCR mixture which contained: 0.2 mM dNTPs, 0.125 unit Amplitaq Gold enzyme (Perkin-Elmer), 1 x buffer II (Perkin-Elmer), 1.5 mM $MgCl_2$ and 100ng mL^{-1} each primer. The 35-cycle PCR was performed as follows: denaturation at 94°C for 1 min, primer annealing at 55°C for 30 s, and an extension step at 72°C for 1 min. The amplified products were analyzed by electrophoresis of 15μL PCR mixture on a 3 : 1 Nusieve gel (FMC Bioproducts, Rockville, MD, USA) plus 0.5 μg mL^{-1} ethidium bromide in Tris/acetate/EDTA buffer (0.04 M Tris/acetate, 0.001 M EDTA, pH 8.0).

Anti-peptide antibody

Methods followed are described in Current Protocols in Immunology [35]. Two peptides were chosen using the software MacVector to predict antigenicity. The two peptides were custom synthesized (Auspep, Melbourne) and conjugated to diptheria toxin (Auspep, Melbourne). Rabbits were immunized with both peptides and serum collected at time zero and after each injection (IMVS, Adelaide.

The two peptides used were:

PEPTIDE Name: TEDDA-N
SEQUENCE: CTGYTERYMGHPDQNEQG-NH2

This is amino acids 773 to 789, plus a Cys at the N-

terminus.

PEPTIDE Name: TEDDR-C
SEQUENCE: GKPYDLQIYPQERHSC-NH2

This is amino acids 836 to 850, plus a Cys at the C-terminus.

These sequences were taken from the C-terminal portion of DPP8.

Monoclonal antibody to DPP8

Standard methods were used for antibody production [35]. Mice were immunized with 2 $x10^7$ live COS-7 (African Green Monkey Kidney) cells that had been transiently transfected with the DPP8 cDNA in the pcDNA3 vector. The final immunisation was with CHO (Chinese Hamster Ovary) cells stably transfected with DPP8 cDNA in the pEE14 vector. Spleen cells were fused with a standard fusion partner, X63Ag8 myeloma cells. Hybridoma culture supernatants were tested by immunoperoxidase histochemistry on monolayers of the DPP8-transfected CHO cell line, using untransfected CHO cells as the negative control. Hybridomas that produced antibody activity were cloned.

RESULTS

Molecular cloning and sequence analysis of DPP8

The insert in ATCC EST AA417787 was 795 bp in length, containing 527 bp of coding sequence, a TAA stop codon and 258 bp of 3′ noncoding sequence (Figure 1).

The hybridization of the Master RNA blot revealed that the gene comprising ESTAA417787 has ubiquitous tissue expression, with high levels of expression in testis and placenta. Based on this expression pattern, a placental

cDNA library was screened with a 484 bp PCR product produced by the forward and reverse DPP8 primers. Sequence homology analysis revealed that only 2 of 23 clones contained 5′ sequence additional to the sequence of ESTAA417787. These cDNA clones were designated T8 and T21, and were 1669 bp and 1197 bp respectively (Figure 1). In addition, comparison of these sequences to ESTAA417787 revealed that T8 cDNA lacked a 153 bp (51aa) region that was present in T21 cDNA and ESTAA417787. This deletion would result in the loss of the catalytic serine (GWSYGG) in T8 cDNA. Many of the other clones characterized appeared to contain unrelated sequence which are probably intronic sequences as a result of incomplete splicing.

The 5′ RACE technique was utilized on both ATC RNA and placental RNA to obtain the 5′ end of the DPP8 gene. The RACE product obtained from activated T cell RNA was 0.2 kb larger than that from placental RNA but otherwise identical (Figure 1). The first methionine within a Kozak sequence was found 214 bp from the 5′ end of the activated T cell RACE product. This 5′ 211bp region was 70.5 % GC rich and contained a number of potential promoter and enhancer elements (Sp1, Ap1 and ETF sites) and so was deduced to be the 5′ flanking region of the DPP8 gene. In order to confirm the identity of the 5′ RACE product as the 5′ end of DPP8, RT-PCR was carried out to span across the junction between the RACE product and T8 cDNA library clone. The RT-PCR on ATC RNA produced two clones ATCd3-2-1 and ATC3-3-10 (Figure 1). Compared to T8 and T21, both clones had an additional insert region of 144bp (48 aa) immediately adjacent to the splice site of T8. Sequence homology analysis of this additional insert region found a homologous region in both the *C. elegans* homologue and DPP4. This clearly showed that T8 and T21 library clones represented splice variants of DPP8. The smaller clone

ATCd3-3-10 was also found to represent another splice variant of DPP8 as it contained a 516 bp deletion at the 5′ end which would result in a deletion of 175 aa.

A full-length DPP8 clone was created using the larger RACE product, ATC3-2-1 and the T21 library clone. This generated a putative DPP8 cDNA of 3.1 kb (including 5′ and 3′ untranslated regions) with an open reading frame of 882 aa for further sequence analysis and examining DPP8 function. This 882 putative DPP8 protein contained no N-linked glycosylation sites and Kyte-Doolittle hydrophobicity analyses revealed it lacked a transmembrane domain, unlike DPP4, FAP and DPP6. Thus it is likely that DPP8 is a cytoplasmic protein (Figure 2). The predicted DPP8 protein shared 51 % amino acid similarity and 27 % amino acid identity with human DPP4; the C termini of these proteins exhibited the most homology (Figure 3).

<u>Tissue distribution of DPP8 as determined by Master RNA and Northern Blot</u>

A master RNA blot was probed with a 484 nt PCR product produced by the forward and reverse DDP8 primers as mentioned previously. The mRNA tissue expression of DPP8 was ubiquitous in all human adult and fetal tissues. A similar ubiquitous expression pattern was observed using DPP4 cDNA as a probe (data not shown). However, by visual assessment the greatest levels of expression using each gene specific probe were in different tissues. The most intense signals using the DPP8 probe were in testis followed by placenta whereas the most intense signals using the DPP4 probe were in salivary gland and prostate gland followed by placenta (data not shown). The probes did not bind any of the negative controls on the blot.

Northern blot analysis was performed on mRNA derived from

different human tissues (Figure 4). Two DPP8 specific probes indicated the presence of transcripts in all tissues examined. A transcript approximately 3.0 kb in size consistent with the approximate expected size of DPP8 message was detected only in the testis. However, two transcripts of 8.0 and 5.0 kb respectively were present in testis, spleen, peripheral blood leukocytes and ovary at high levels; in prostrate, small intestine, and colonic mucosa at moderate levels; and in the thymus at lower levels. The Multiple tissue Northern blot was also probed with radiolabeled human β-actin probe and a common 2.0 kb transcript was seen in all tissues (Figure 4).

<u>Expression of DPP8 in mice determined by Northern Blot and rtPCR.</u>

The human DPP8 cDNA sequence cross-hybridized with murine derived liver RNA. The Northern blot containing total RNA from mouse liver hybridized to a human DPP8 probe, showing that DPP8 mRNA is expressed in mouse liver (Figure 9A). Two mRNA transcripts of murine DPP8 were present. This is a similar pattern to that observed for human DPP8. These transcripts probably represent different length 5′ and 3′ untranslated regions of the murine DPP8 gene. The presence of DPP8 mRNA in the mouse liver was also demonstrated using rt-PCR. The primers tested generated a 537bp PCR product. A Southern blot of this product confirmed that the murine DPP8 cross-hybridizes with human DPP8 (Figure 9B).

<u>Expression and functional activity of DPP8</u>

To assess the function of DPP8 protein, the full length DPP8 cDNA of 3.1 kb was cloned into the *Xba I* site of pcDNA3.1A/V5/His expression vector to produce two constructs. The first construct, pcDNA3.1-DPP8, expressed DPP8 protein on its own whilst the second construct,

pcDNA3.1-DPP8/V5/His expressed a protein with the V5 epitope and His tag fused to the C-terminus of DPP8 to facilitate analysis of protein expression. Mammalian expression constructs were stably transfected into COS-7 cells and cellular sonicates prepared. Consistent with the molecular weight predicted from the amino acid sequence a 100 kDa monomer was detected by Western blotting of stable DPP8/V5/His expressing cells (Figure 6). DPP8/V5/His protein was detected in the cytoplasmic compartment but not on the surface of ethanol fixed stable DPP8/V5/His expressing COS cells, using the anti-V5 mAb.

DPP8 is a dipeptidyl peptidase

Sequence homology between DPPIV and DPP8 suggested functional similarities, so cell lysates of DPP8-transfected cells were examined for proline-specific peptidase activity. DPPIV expressed in COS-7 cells with or without the V5/His tag were positive controls, and negative controls included vector-only transfected COS07 cells. Extracts of DPP8-transfected COS-7 cells hydrolyzed H-Ala-Pro-pNA and H-Arg-Pro-pNA but not H-Gly-Pro-pNA, H-Gly-Arg-pNA, H-Gly-Pro-toluenesulfonate or H-Gly-Pro-7-amino-4-trifluoromethylcoumarin above the levels exhibited by untransfected COS-7 cells (data not shown). The pH optimum of DPP8 enzyme activity was 7.4 (Fig. 5A), similar to the pH 7.8 optimum DPPIV enzyme activity [43,44]. DPP8 exhibited little activity below pH 6.3, suggesting that it is not an enzyme of the lysosome/endosome compartment. Of all the substrates tested on cell monolayers, only Ala-Pro-4MβNA/HCl stained DPP8-transfected COS cells and CHO cells (data not shown).

Both purified recombinant DPP8/V5/His and purified recombinant DPPIV/V5/His hydrolyzed H-Ala-Pro-pNA, G-Gly-Pro-pNA and H-Arg-Pro-pNA. Transfection with DPP8

possibly causes increased dipeptidase, tripeptidase and endopeptidase activities, similar to an effect of DPPIV transfection of melanoma cells [18]. Indeed, our results showed that DPP8 transfected COS-7 cells, but not purified recombinant DPP8, exhibited tripeptidyl peptidase activity using the substrate H-Ala-Ala-Pro-pNA and endopeptidase activity using the substrate Z-Ala-Pro-pNA (data not shown). This was investigated further, and neither of the tripeptidyl peptidase substrates H-Ala-Ala-Phe-pNA or H-Ala-Phe-Pro-pNA [45] nor the prolyl endopeptidase substrates Z-Ala-Pro-pNA or succinyl-Ala-Pro-pNA were cleaved by purified DPP8. Our data clearly demonstrate that DPP8 is a dipeptidyl peptidase and lacks tripeptidyl peptidase or endopeptidase activities.

The nature of the catalytic mechanism of DPP8 was further investigated using various inhibitors. DPP8 enzyme activity was significantly inhibited by serine proteinase inhibitors and was insensitive to inhibitors of metalloproteinases, aspartyl proteinases and cysteine proteinases. DPP8 enzyme activity was significantly inhibited by zinc, which completely inhibits DPPIV enzyme activity [46]. The peptides Ala-Pro-Gly and Lys-Pro mimic DPP8 substrates and probably competitively inhibited DPP8.

Chromosomal localization of DPP8

Two probes were used for FISH analysis, ESTAA417787 and the T8 clone from the placental library. Seventeen metaphases from the first normal male were examined for fluorescent signal. All of these metaphases showed signal on one or both chromatids of 15 at band q22 (Figure 5). There were a total of 2 non-specific background dots observed in these metaphases. A similar result was obtained from the hybridization of the probe to 15 metaphases from the second normal male (data not shown).

Analysis of *DPP8* gene expression by RT-PCR

DPPIV is expressed by most lymphocytes and lymphocytic cell lines but upregulated on activated lymphocytes [47, 41, 48, 49]. The various splice variants of DPP8 might not encode functional protein, so the PCR was designed to detect only mRNA that contained full-length sequence (Fig. 1). At 35 cycles, amplification product of the expected size (783 bp) was readily observed in OKT3-stimulated PBMCs (six of six subjects; Fig 8) but not in unstimulated PBMCs from most subjects (four of five, Fig. 8A), suggesting that more DPP8 mRNA is expressed in activated T cells than in unstimulated PBMCs. Similar RT-PCR data were obtained from PBMCs stimulated with phytohaemagglutinin (data not shown). In addition, DPP8 mRNA was expressed in all B and T cell lines examined and in both liver and placenta(Fig. 8B).

Anti-peptide antibody

The sera of two rabbits were tested by ELISA in peptide-coated wells. Both sera bound both peptides whereas the pre-immunisation serum samples did not exhibit specific binding. Western blots on extracts of cell lines, cell lines transfected with DPP8 cDNA and activated human lymphocytes showed that a rabbit antiserum to the two DPP8 peptides binds a 100kDa band, which is the size of DPP8. (Data not shown).

Table 1. K_m and V_{max} values for DPP8 and DPPIV

	K_m (mM)		V_{max} (ΔA min^{-1} x 1000)	
	DPPIV	DPP8	DPPIV	DPP8
H-Ala-Pro-pNA	0.374 ± 0.134	0.991 ± 0.171	9.6 ± 1.0	12.4 ± 0.9
H-Gly-Pro-pNA	0.347 ± 0.088	0.467 ± 0.064	7.2 ± 0.49	3.5 ± 0.14

Table 2. Inhibition of the peptidase activity of DPP8 in comparison with DPPIV. Common proteinase inhibitors of various enzyme types were incubated with the purified peptidases before assay with the substrates H-Ala-Pro-pNA on DPP8 or H-Gly-Pro-pNA on DPPIV. AEBSF, 4-(2-aminoethyl)benzenesulfonylfluoride.

Type of inhibitor	Concentration	Residual activity (% of control) DPP8	DPPIV
None		100	100
Serine proteinase			
AEBSF	4 mM	40	52
Aprotinin	4 µg mL^{-1}	47	81
Benzamidine/HCl	10 mM	82	89
Peptides			
Gly-Gly-Gly	10 mM	99	106
Ala-Pro-Gly	10 mM	51	67
H-Lys-Pro-OH HCl salt	4 mM	63	45
Zinc sulphate	2 mM	25	0
Metalloproteinase			
EDTA	2 mM	115	99
Aspartate(acidic) proteinase			
Pepstatin	2 µg mL^{-1}	107	110
Leupeptin	0.1 mM	93	104
Cysteine(thiol) proteinase			
Iodoacetamide	2 mM	100	115
Dithiothreitol	2 mM	108	109

Discussion

We describe the cloning, recombinant expression, biochemistry and tissue expression of a novel human DPPIV-related postproline peptidase that we have named DPP8. DPP8 exhibited dipeptidyl aminopeptidase but not tripeptidyl peptidase or endopeptidase activity. Like DPPIV, DPP8 was found to exhibit significant mRNA expression in activated T cells. Clear indications that DPP8 is a monomeric, nonglycosylated, soluble, cytoplasmic protein, which are characteristics of PEP but not of DPPIV, FAP or DPP6, were provided by our sequence and localisation data. DPP8 enzyme activity had a neutral pH optimum, suggesting that it is not active in the acidic lysosome/endosome compartment.

By homology with DPPIV, DPP8 is a member of the DPPIV-like gene family, a member of the prolyl oligopeptidase family S9b, and a member of the enzyme clan SC. The residues in DPP8 that potentially form the charge-relay system are Ser739, Asp817 and His849 (Fig.2). The dipeptidyl peptidase activity of DPP8 and the absence of detectable tripeptidyl peptidase or endopeptidase activities by purified DPP8 further support its placement in the S9b family. Furthermore, the DPP8 substrate specificity was indistinguishable from that of the structurally related peptidases DPPIV and FAP.

The role of DPPIV in human lymphocytes has been studied in detail using enzyme inhibitors [49, 50-54]. DPPIV-specific inhibitors suppress both DNA synthesis and cytokine production *in vitro* [48, 49, 52]. In addition, DPPIV-specific inhibitors decrease phorbol myristate acetate-induced tyrosine phosphorylation in human lymphocytes, further suggesting a role for DPPIV enzyme

activity in lymphocyte activation [54]. *In vivo*, inhibitors of DPPIV suppress arthritis [20] and prolong cardiac allograft survival in animal models [55]. The ability of DPP8 to cleave DPPIV substrates indicates that DPPIV inhibitors may also inhibit DPP8 and that inhibitor studies may require further interpretation. Indeed, DPP8 may be responsible for some of the physiological functions that have been assigned to DPPIV.

FAP and DPPIV are integral membrane glycoproteins and require dimerization for catalytic activity [9, 56, 57]. In contrast, DPP8 and PEP are non-glycosylated cytosolic proteins that are catalytically active as monomers [58] and cleave Pro-Xaa bonds [43,59]. However, the substrate specificity of PEP is distinct from DPP8. PEP is an endopeptidase that does not cleave if a free α-amine lies N-terminal to the proline (e.g. it does not cleave H-Ala-Pro). Recently we have proposed that the tertiary structure of DPPIV is similar to that of PEP in having a seven-blade β-propeller domain and an α/β-hydrolase domain [3, 39, 1]. The significant sequence identity between DPP8 and DPPIV indicates that the tertiary structures of DPP8 and DPPIV are similar. However, DPP8 contains 110 amino acids more than DPPIV, so it could have an additional element of tertiary structure such as an eighth propeller blade.

The ancestral relationships between DPP8, DPPIV and FAP are reflected in their chromosomal localization. While DPPIV and FAP have both been localized to the long arm of chromosome 2, 2q24.3 [60] and 2q23 [61] respectively, DPP8 was localized to 15q22. The related genes DPP6 and PEP have been localized to chromosome 7 [62] and 6q22 respectively [63].

Two human disease loci have been mapped to 15q22. These loci are an autosomal recessive deafness locus [64] and a form of Bardet-Biedl syndrome, type 4 [65]. Two of the clinical manifestations of Bardet-Biedl syndrome are obesity and diabetes. Attractin [66] and DPPIV have roles in obesity [67] and diabetes [22, 68, 15] respectively and as their substrate specificities overlap with that of DPP8, it is possible that DPP8 may be involved in Bardet-Biedl syndrome.

DPPIV is expressed on the surface of T cells and is a costimulatory molecule called CD26 [3]. CD26-negative cell lines have residual DPPIV enzyme activity and PBMC have non-DPPIV derived activity against Ala-Pro substrates [69], indicating the existence of other peptidase(s) with DPPIV-like activity. DPPIV-β exhibits a peptidase activity similar to DPPIV but is a 70-80 kDa cell surface glycoprotein [70] and is therefore distinct from DPP8.

The biological significance of the three splice variants of DPP8 that we discovered is unknown. None of these splice variants result in a frame shift or premature protein termination (Fig. 1). Two of the splice variants contain all the predicted catalytic triad residues and thus potentially produce proteins with peptidase activity. Alternate splice forms of FAP mRNA have also been observed [71, 72]. It is possible that expression of splice variants may be used to regulate the levels of active protein. DPP8 Northern blots revealed a number of differently sized transcripts. The predicted sizes of splice variants of DPP8 ranged from 2.6 to 3.1 kb whereas the large transcripts seen in most tissues examined in the Northern blots were 8.5 kb and 5.0 kb respectively. Similarly, two other members of the DPPIV-like gene family, DPPIV and DPP6, exhibit mRNA transcripts in

Northern blots that are much larger than the cDNA size [60, 61]. We propose that the major transcripts for DPP8 mRNA and its splice variants lie within the 5 kb band while the 8.5 kb transcript(s) may contain additional 5' and 3' untranslated sequences. DPP8 appears to be like DPPIV in having a ubiquitous mRNA expression pattern by Northern analysis while being upregulated in activated T cells. The similarities between DPP8 and DPPIV suggest that DPP8 may, like DPPIV, play a role in T cell costimulation and proliferation. The development of DPP8 specific antibodies or inhibitors will facilitate work in this area.

In summary, we have identified and characterized a novel human dipeptidyl aminopeptidase DPP8 with structural and functional similarities to DPPIV and FAP. With many diverse biological roles suggested for DPPIV, particularly in the immune system, and the roles of FAP in tumor growth and liver disease, it will be interesting to investigate the roles of this new member of the DPPIV-like gene family in these systems. Further work in understanding this novel protein and the elucidation of inhibitors and physiological substrates will help identify the specific functions of individual members of this gene family.

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